Strategies to enhance the activity of key enzymatic steps of Isoprenoid Biosynthesis in *Saccharomyces cerevisiae*

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Certificate of Examination

This is to certify that the dissertation titled 'Strategies to enhance the activity of key enzymatic steps of Isoprenoid Biosynthesis in *Saccharomyces cerevisiae'* submitted by **Mr Anuthariq A V (Reg. No. MS16119)** for the partial fulfillment of the BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Shravan Kumar Mishra Dr. Rachna Chaba Prof. Anand Kumar Bachhawat

(Supervisor)

Dated: May 30, 2021

Declaration

The work presented in this dissertation has been carried out by me under the guidance of **Prof. Anand Kumar Bachhawat** at the Indian Institute of Science Education and Research, Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Anuthariq A V

May 30, 2021

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. Anand Kumar Bachhawat

(Thesis supervisor)

Acknowledgement

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I dedicate this work to my parents, to whom I will be indebted always. I cannot thank them enough. Their constant support, faith, and encouragement are invaluable, and their teachings have helped me move through life.

Anuthariq A V

List of figures

List of tables

Abbreviations

G6PD	glucose 6-phosphate dehydrogenase
MVP	Mevalonate Pathway
PPP	Pentose Phosphate Pathway
NADPH	nicotinamide adenine dinucleotide phosphate(Reduced)
GGPPS	Geranylgeranyl Pyrophosphate synthase
GGPP	Geranylgeranyl Pyrophosphate
$NADP^+$	nicotinamide adenine dinucleotide phosphate
R5P	Ribulose 5-phosphate
6-PG	6- Phosphogluconate
6-PGL	6-phosphogluconolactonase
6PGDH	6-phosphogluconate dehydrogenase
GSH	Glutathione
GSSG	Oxidized glutathione
ROS	Reactive oxygen species

CONTENTS

Abstract

Mevalonate pathway (MVP) is an essential metabolic pathway present in eukaryotes, archaea, and some bacteria[1]. MVP is responsible for synthesizing a diverse class of biomolecules, that involves isoprenoids, cholesterol, and a precursor to many commercially valuable terpenoids. The efforts to exploit this pathway for value-added product biosynthesis by expressing the genes in heterologous hosts is being attempted by different groups. Isoprenoids produced from genetically engineered organisms, using renewable carbon source, are the new micro-factories for many sustainable chemical productions. Their capability to replace petroleum-derived production strategies for various terpenoids is a step towards sustainable development[2]. However, for the synthetic biological route to succeed, a very efficient production system with an efficient synthesis pathway is required, which is why efforts to enhance the biosynthetic pathways are extensively being worked upon. In MVP, converting HMG Co-A into mevalonate is a rate-limiting step that requires two molecules of NADPH for reduction. In this thesis, we have attempted to increase the flux by increasing the reaction substrate NADPH by the creation of gene fusions (synthetic metabolon) in the Pentose phosphate pathway, which is a key cytosolic NADPH production pathway. Geranylgeranyl Pyrophosphate (GGPP) is a crucial metabolic hub where the flux is diverted to different branching pathways. In the second part of the thesis, there was an attempt at engineering the GGPP synthase enzyme in order to increase the turnover rate of GGPP production.

Chapter 1: Introduction

1.1 The Mevalonate pathway and isoprenoid biosynthesis

A large number of commercially important terpenoids are sourced from plants. Extraction from plants is the classic way to get there biomolecules, which gives unreliable yields. Further, these have complex chemical structure and are thus also chemically difficult to synthesize. This throws light on the inadequacy of chemical synthesis and plant extraction methods for obtaining these compounds. Thus researchres are turning towards utilizing the potentials of genetically modified organisms[2]. The Mevalonate pathway is responsible for the synthesis of about 40,000 biomolecules in living cells. In humans, the Mevalonate pathway is involved in multiple cellular processes by synthesizing isoprenoids including cholesterol, dolichol, heme-A, isopentenyl tRNA, and ubiquinone[1], which is why it is a clinically well-studied pathway and several drugs are targeted to this pathway. In the case of plants, isoprenoids like chlorophylls, tocopherols, phylloquinone, gibberellins, and carotenoids[3] are synthesised from MVP products as precursors.

1.1.1 Yeast Mevalonate Pathway

For the production of many valuable isoprenoids, mainly *Saccharomyces cerevisiae* and *Escherichia coli* are employed. Even though a slower growth and a lesser metabolite production are observed in *S. cerevisiae*, they could produce more structurally diverse chemicals. Besides, when compared to *E. coli, S. cerevisiae* is more robust in large-scale fermentations, relatively tolerant to low pH and high concentrations of sugars, and fairly resistant to inhibitors. Besides, advanced yeast genetics tools available for *S. cerevisiae* makes it a better option for industrial purpose[2]. Yeast and other fungus utilize MVP for isoprenoid precursor production. The pathway starts with the consumption of Acetyl CoA to produce DMAPP and IPP, where the addition of IPP units to DMAPP

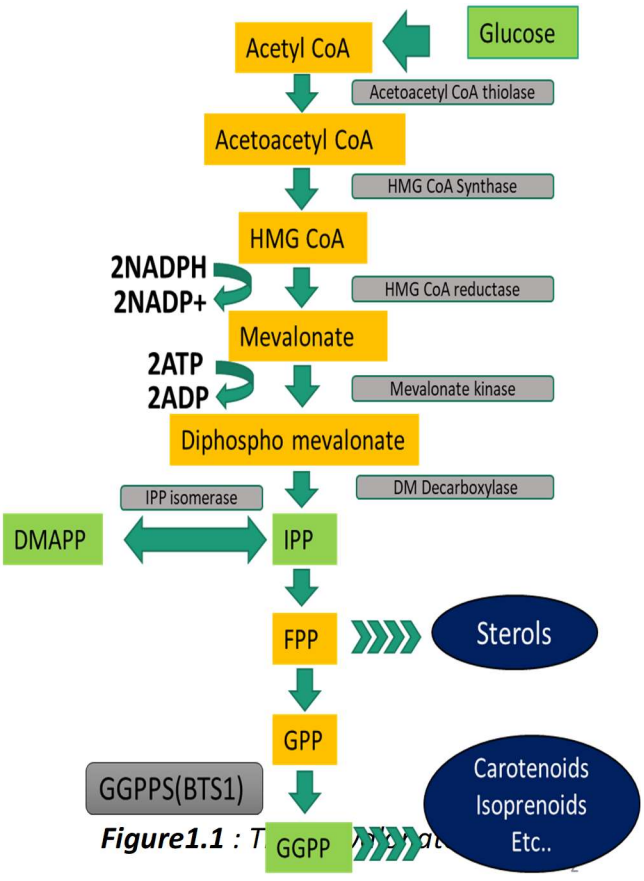


***Figure1.1** : T*

produces short-chain prenyl diphosphates that serve as building blocks of all isoprenoids (Fig. 1.1).

Recent advances in targeting individual steps and enzymes in isoprenoid biosynthetic pathway have pushed the efficiency far but still not enough. Thus the need to enhance the efficiency of the pathway is inevitable.

1.2 Pentose Phosphate Pathway (PPP): Major NADPH source

A rate-limiting step in MVP is the reduction of HMG CoA to mevalonate which requires two molecules of NADPH. The question of increasing NADPH for improving MVP links the pentose phosphate pathway (PPP) with it. The PPP, also known as hexose monophosphate shunt (HMS), is a central metabolic pathway that runs parallel to glycolysis. The major role of the oxidative branch of PPP is the production of reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH) and nucleotide precursors like ribulose 5-phosphate (R5P)[4] (Fig. 1.2). The cell survival during oxidative stress depends on the oxidative stress response mediated by Glutathione (GSH), which reduces ROS and itself gets oxidized to GSSG. To combat oxidative stress, the cell regenerates GSH from GSSG using NADPH as the reducing agent[5].

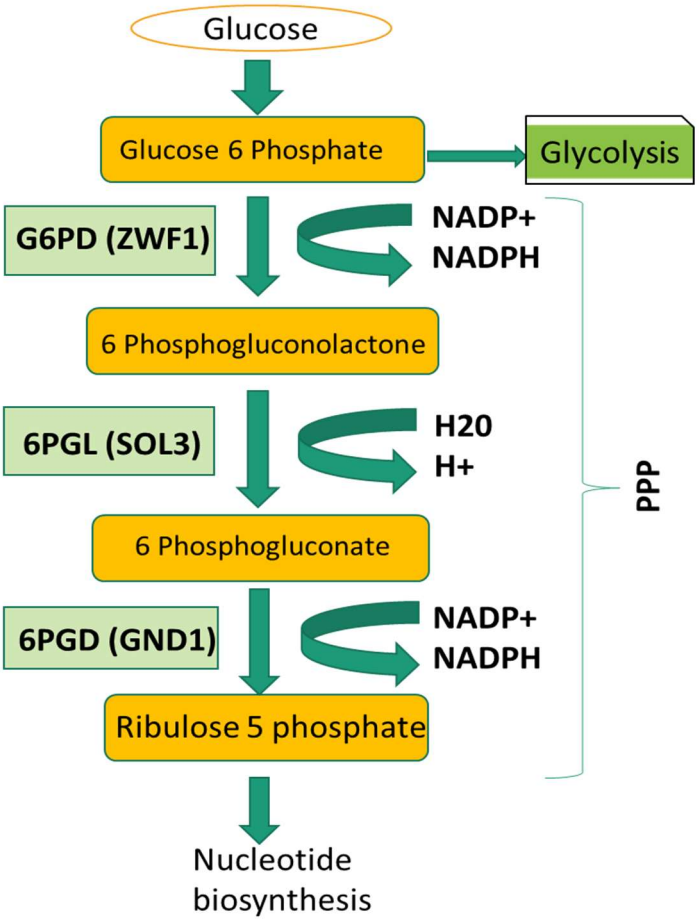


Figure 1.2: schematic representation of pentose phosphate pathway with enzymes responsible, showing NADPH production.

The key enzymes acting in the oxidative branch of PPP are G6PD encoded by ZWF1 gene (NADPH generating), 6PGL encoded by SOL3 gene, and 6PGD encoded by GND1 gene (NADPH generating).

Table 1.1 shows reaction and products in PPP.

Reactants	Products	Enzyme
Glucose 6-phosphate + $NADP^+$	6-phosphoglucono-δ-lactone+ **NADPH**	Glucose-6-phosphate dehydrogenase (G6PD)
6-phosphoglucono-δ-lactone + H_2O	6-phosphogluconate + H^+	6-phosphogluconolactonase(6PGL)
6-phosphogluconate + $NADP^+$	ribulose-5 phosphate + **NADPH** + CO_2	6-phosphogluconate dehydrogenase (6PGDH)

***Table* 1.1:** Reactions in Oxidative PPP

1.2.1 Fusion proteins in pentose phosphate pathway

Fusion proteins consist of at least two domains that are encoded by separate genes joined so that they are transcribed and translated as a single unit, producing a single protein. They are created naturally by chromosomal rearrangement events and artificially by recombinant DNA techniques. Fusion proteins are mainly created for purification of cloned genes, as reporters of expression level, and as histochemical tags to enable protein visualization. The concept of metabolon is not new in synthetic biology. The term metabolon is used to describe complexes of enzymes that carry out sequential reactions. They are expected to increase the efficiency of metabolic pathways by channelling substrates between enzymes. Metabolons may increase flux by increasing the local concentration of intermediates, decreasing the concentration of enzymes needed to maintain a given flux, directing the products of a pathway to a specific subcellular location, or minimizing the escape of reactive intermediates[6].

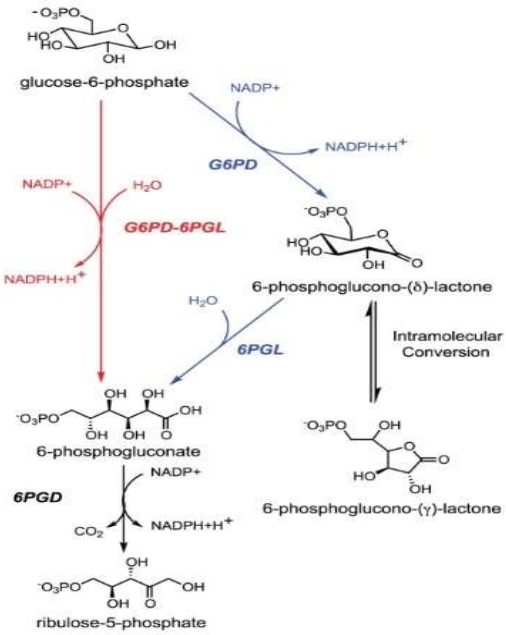


***Figure 1.3**: The route taken by organisms with fusion enzymes is shown in red, while the route taken by other organisms with a normal functioning pentose phosphate pathway is shown in blue. figure reproduced from Stover, N. A 2011* [7].

In the PPP, glucose-6- phosphate is converted into 6-phosphoglucono-(δ)-lactone by Glucose-6-phosphate dehydrogenase (G6PD) and it further undergoes spontaneous hydrolysis to form 6-phosphogluconate with the help of 6-

phosphogluconolactonase (6PGL) which is required for the efficient functioning of the pathway. In the absence of 6PGL, the δ-lactone will undergo a conversion to the γ-lactone form, which does not undergo spontaneous hydrolysis and builds to toxic levels in the cells. The 6PGL enzyme thus ensures that the δ-lactone product of G6PD will be hydrolyzed rapidly, before its conversion to the γ-lactones[7]. Thus an efficient transfer intermediate is required and can be possible by metabolon formation (Fig. 1.3). There is evidence of fusion protein with the first two enzymes of PPP in the malarial parasite, Plasmodium, which requires high levels of NADPH, crucial for oxidative stress defence from host immune response. The activity of these fusion proteins relative to non-fusion was found to be superior in terms of the turnover rate, but not substrate G6P affinity[8]. Another computational study in search for Fusion proteins in PPP in the whole phylogenetic tree found that there occurred three independent fusion events in evolution for different clades, on their PPP enzymes. The orientations and combination of enzymes vary in the groups but the fusion event seems selected throughout time[7] (Fig. 1.4).

Thus, throwing light on the question of fusions can conceivably increase the NADPH production via product channelling, and that can be a reason for the selection of independent fusion events in PPP.

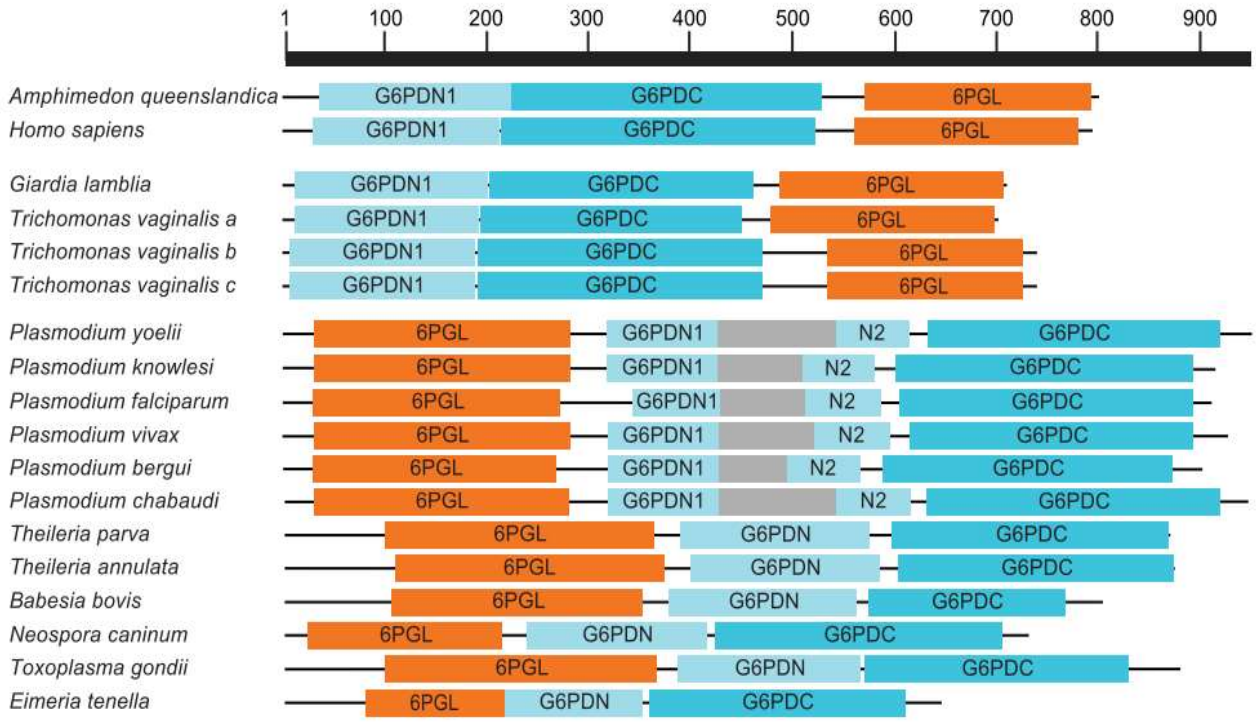


Figure 1.4: *Fusions of the G6PD (N and C terminal regions) and 6PGL genes found in excavates, apicomplexans, and two representative G6PD/6PGL fusions in metazoans. Figure reproduced from Stover, Dixon, and Cavalcanti, 2011.*

To address this question in *S. cerevisiae*, we created a fusion product of G6PD, encoded by ZWF1 gene and 6PGL encoded by SOL3 gene with a suitable linker selected from *Trichomonas vaginalis* because of its smaller size, naturally occurring fusion with least insertion, and fusion gene similarity with *S. cerevisiae*. The fusion proteins are created in both orientations: N-terminal ZWF1 and C-terminal SOL3 and N-terminal SOL3 with C-terminal ZWF1 along with a His-tag (Fig. 1.5) for protein purification followed by an in-vitro enzyme kinetic study.

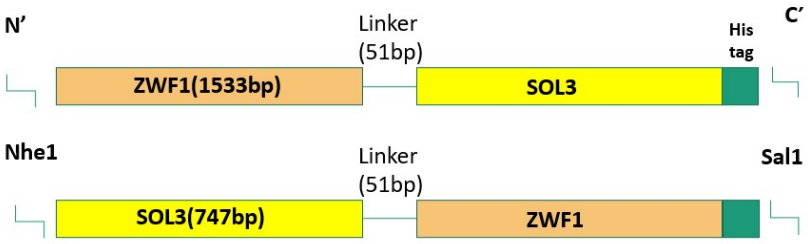


***Figure 1.5**: Fusion proteins of ZWF1 and SOL3*

In-vivo expression studies are done by complementation in Δzwf1 cells, which are not able to synthesize methionine. This methionine auxotrophy is believed to be due to insufficient production of NADPH which is required in the methionine biosynthesis pathway[9]. The fusion proteins were later expressed in *E. coli* protein expression system BL21 DE3 pLys-S cells and induced using IPTG, and purified by Ni-NTA for in vitro activity studies.

1.3 GGPP: A central pathway hub to many branching pathways

Geranylgeranyl pyrophosphate (GGPP) is a key metabolic hub where the MVP flux is diverted to many different pathways (Fig. 1.6). GGPP is synthesized from GPP, by GGPP synthase (GGPPS) encoded by

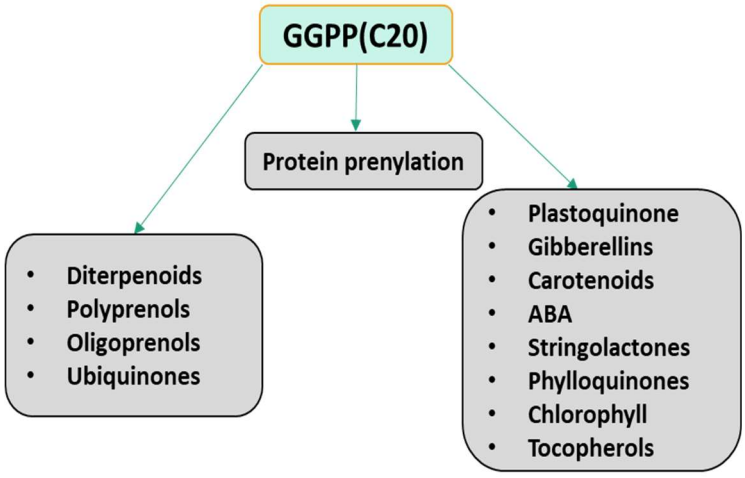


***Figure 1.6**: Pathways into which GGPP pool is utilized.*

the BTS1 gene in *S. cerevisiae*. Another GGPPS (Rt GGPPS) from *Rhodosporidium toruloides,* an oleaginous yeast, with high lipid accumulation in intracellular lipid bodies[10], gives them red colour because of high carotenoid production, shows higher activity than BTS1(Sc GGPPS); this Rt GGPPS cloned and expressed in *S. cerevisiae* shows a higher GGPP flux output, studied by a carotenoid screening method in previous work.

The enhanced activity of Rt GGPPS raises a question of the structural and functional difference of Rt GGPPS and Sc GGPPS. To investigate this, we did a preliminary analysis of sequence comparison by multiple sequence alignment on GGPPSs of oleaginous yeasts along with Rt GGPPS and Sc GGPPS. From the crystal structure data known for the human and Sc GGPPS[11,12], we could identify key residues which are conserved in all other oleaginous yeasts but not in Sc GGPPS. The amino acids are shortlisted by the most structural and functional difference they show, and their location in important activity site or domain in comparison to Sc GGPPS and other GGPPS[11,12].

Since the crystal structure of Rt GGPPS was not solved up till now, we used a computational modelling approach to model and study the effect of potential residues involved in the GGPPS catalysis both *in-silico* and experimentally.

Chapter 2: Materials and methods

SECTION A: Materials

2.1 Chemicals and Reagents

All chemicals used were obtained from commercial sources and were of analytical grade. Media components, fine chemicals, and reagents were purchased from Sigma Aldrich (St. Louis, USA), HiMedia (Mumbai, India), Merck. India Ltd (Mumbai, India), USB Corporation (Ohio, USA), or Difco, USA. Oligonucleotides (primers) were designed using SnapGene (version 2.4.3) software and were purchased from Integrated DNA Technologies (IDT). Enzymes (Restriction enzymes, T4 DNA ligase, Calf Intestinal Phosphatase (CIP), Antarctic phosphatase, *Vent* DNA polymerase, *Phusion* High-fidelity DNA polymerase, and other modifying enzymes), their buffers, dNTPs, DNA, and protein molecular weight markers were purchased from New England Biolabs Inc, (Beverly, MA, USA) or Thermo Scientific. Gel-extraction kits and plasmid miniprep columns were obtained from BioNEER and Promega. GSH was obtained from Sigma-Aldrich, USA.

2.2 Strains and Plasmids

Escherichia coli DH5α is used as the cloning host and *E. coli* BL21(DE3)Plys(S) is used for protein expression by IPTG induction. The genotype for the *E. coli* and *Saccharomyces cerevisiae* strains used in the study are given in Table 2.1. The list of various plasmids used in this study is given in Table 2.2.

Table 2.1:List of bacterial and yeast strains used in the study

Strain	**Genotype**	**Source**
*Escherichia coli s*trains		
ABE 460 (DH5α)	F $^{-}$*gyr A96(Nal) recA1 relA1 endA1 thi-1 hsdR17*($r_k^- m_k^+$) *gln V44 deoR Δ(lacZYA-argF) U169 [ϕ80dΔ(lacZ) M15]*	Lab strain
Xxx (BL21(DE3) pLysS)	BF– ompT gal dcmlonhsdSB(rB–mB–) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]K-12(λS) pLysS[T7p20 orip15A](CmR)	Lab strain
Saccharomyces cerevisiae strains		
ABC 733 (BY4741)	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Lab stock
ABC 5165	*Δzwf1* in BY4741;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; YNL241C::KanMX4*	Euroscarf
ABC 6069	*Δbts1* in BY4741;*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; YHR183W::KanMX4*	Euroscarf
ABC XXXX (Int Cen.PK)	*MAT*a/α *ura3-52/ura3-52 trp1-289/trp1-289 leu2-3,112/leu2-3,112 his3 Δ1/his3 Δ1 MAL2-8C/MAL2-8C SUC2/SUC2 with the integrated GFP sensor*	Lab stock

Table 2.2: List of plasmids used in the study

Plasmid name	**Clone no.**	**Description**

Plasmids used in the study		
p ET 23a vector		
p416TEF vector	ABE 443	The CEN-vector bearing URA3 marker and TEF Promoter-MCS-terminator for yeast expression and Amp^{r}marker for selection in *E. coli*. (Mumberg, et al., 1995)
pRS313TEF	ABE 3569	The CEN-vector bearing HIS3 marker and TEF promoter-MCS- terminator for yeast expression and Amp^r marker for selection in *E. coli*. Vector was constructed by excising TEF promoter and MCS from pRS416TEF using SacI and ApaI site and cloned at pRS313 vector at SacI and ApaI site.
pRS416TEF-PS(AT)+PD	ABC 5856	Tef PD with CYC terminator is amplified and cloned in the KpnI site of pRS416TEF PS(AT)
Plasmids constructed during the study		
pRS313TEF-WT BTS1	ABE6098	BTS1 cloned from genomic DNA of *S. cerevisiae* in pRS313TEF between BamHI and XhoI sites.
pRS313TEF-M1 BTS1 (I46F)	ABE6099	Mutant BTS1 with mutation I46F cloned in pRS313TEF between BamHI and XhoI sites.
pRS313TEF-M2 BTS1 (S70A)	ABE6100	Mutant BTS1 with mutation S70A cloned in pRS313TEF between BamHI and XhoI sites.
pRS313TEF-M3 BTS1 (T889+S90A)	ABE6101	Mutant BTS1 with mutations T889+S90A cloned in pRS313TEF between BamHI and XhoI sites.
pRS313TEF-M4 BTS1 (S98Q)	ABE6102	Mutant BTS1 with mutation S98Q cloned in pRS313TEF between BamHI and XhoI sites.
pRS313TEF-M5 BTS1 (S193D)	ABE6103	Mutant BTS1 with mutation S193D cloned in pRS313TEF between BamHI and XhoI sites.

pRS313TEF-M1M2-BTS1 (I46F and S70A)	ABE6195	Mutant BTS1 with mutations I46F and S70A cloned in pRS313TEF between BamHI and XhoI sites.
pRS313TEF-M4M5-BTS1 (S98Q and S193D)	ABE6196	Mutant BTS1 with mutations S98Q and S193D cloned in pRS313TEF between BamHI and XhoI sites.
pRS313TEF-MX5-BTS1 (I46F,S70A,T88P,S90A,S98Q and S193D)	ABE6196	Mutant BTS1 with mutations I46F, S70A, T88P, S90A, S98Q and S193D cloned in pRS313TEF between BamHI and XhoI sites.

2.3. Oligonucleotides

The list of various oligonucleotide primers used in this study is given in Table 2.3.

Table 2.3: List of Oligonucleotides (and their sequences) used in this study

Oligonucleotide name	**Sequence (5' to 3')**
SOL3-ZWF1- NheI FP	TCATGCTAGCATGGTGACAGTCGGTGTG
SOL3-ZWF1-6XHIS-SalI RP	TCATGTCGACCTAGTGGTGATGGTGATGATGATTATCCTTCGTATCTTC
SOL3-ZWF1 Linker FP	AAAGTTCAAACGAAAACTTTTTTGGATATAGGCGTTGGGATGATTAAAAGGAACAAAAAGAATCTGGCTAGTGAAGGCCCCGTCAAATTC
SOL3-ZWF1 Linker RP	GAATTTGACGGGGCCTTCACTAGCCAGATTCTTTTTGTTCCTTTTAATCATCCCAACGCCTATATCCAAAAAAGTTTTCGTTTGAACTTT
ZWF1-SOL3-NheI FP	TCATGCTAGCATGAGTGAAGGCCCCGTCAAATTC
ZWF1-SOL3-6XHIS-SalI RP	TCATGTCGACCTAGTGGTGATGGTGATGATGAAAAGTTTTCGTTTGAAC
ZWF1-SOL3 Linker FP	GCCAGAAGATACGAAGGATAATTTGGATATAGGCGTTGGGATGATTAAAAGGAACAAAAAGAATCTGGCTCAAGTGACAGTCGGTGTGTTTTCTG

ZWF1-SOL3 Linker FP	CAGAAAACACACCGACTGTCACTTGAGCCAGATTCTTTTTGTTCCTTTTAATCATCCCAACGCCTATATCCAAATTATCCTTCGTATCTTCTGGC
ZWF1-NheI FP	TCATGCTAGCATGAGTGAAGGCCCCGTCAAATTC
ZWF1-6XHIS-SalI RP	TACTGTCGACCTAGTGGTGATGGTGATGATGATTATCCTTCGTATCTTCTG
BTS1-BamHI FP	TCATGGATCCATGGAGGCCAAGATAGATGAG
BTS1 XhoI RP	TCATCTCGAGTCACAATTCGGATAAGTGG
M1 I48F FP	ACTAAATTTAATAGTTCAATTTAACAGAGTTATGAATTTGC
M1 I48F RP	GCAAATTCATAACTCTGTTAAATTGAACTATTAAATTTAGT
M2 S72A FP	TGTTGAGCTCTTGCATAATGCCAGCCTTTTAATCGACGATA
M2 S72A RP	TATCGTCGATTAAAAGGCTGGCATTATGCAAGAGCTCAACA
M3 T90P, S92A FP	TCCCTTGAGAAGGGGACAGCCCACTGCTCACTTAATCTTCGGTGTAC
M3 T90P, S92A RP	GTACACCGAAGATTAAGTGAGCAGTGGGCTGTCCCCTTCTCAAGGGA
M4 S100QFP	CTTAATCTTCGGTGTACCCCAAACTATAAACACCGCAAATTA
M4 S100QRP	TAATTTGCGGTGTTTATAGTTTGGGGTACACCGAAGATTAAG
M5 S195DFP	TTCCTCACACCACGGCCATGATTTGGTTCCTTTCATAAATC
M5 S195DRP	GATTTATGAAAGGAACCAAATCATGGCCGTGGTGTGAGGAA
Rt GGPPS NheI FP	TCTAGCTAGCATGTCGCTGGACTGGTACGA

Rt GGPPS 6XHIS SalI RP	TCTAGTCGACTCAGTGGTGATGGTGATGATGGACTTTGGG AAGCTCGTGCG
BTS1-Nhei FP	TCATGCTAGCATGGAGGCCAAGATAGATGAG
BTS1-6XHIS-SalI RP	TCTAGTCGACTCAGTGGTGATGGTGATGATGCAATTCGGA TAAGTGGTC

2.4 Media

All the media, buffers and stock solutions were prepared using Millipore elix 5 deionized water unless otherwise mentioned. They were sterilized, as recommended, either by autoclaving at 15 lb/inch^2 (psi) pressures at 121ºC for 15 minutes, or by using membrane filters (Advanced Microdevices Pvt. Ltd., India) of pore size 0.2-0.45 µm (for heat-labile compounds). Additional amino acids, nutrients and, oxidative reagents such as Methylglyoxal and Diamide were prepared as sterile stock and added as per requirements. Agar was added, if required, at a final concentration of 2.2%. the antibiotics were added at a required concentration.

2.4.1. LB Medium (Luria–Bertani) (per 1000 mL)	Yeast extract 5 g/L Tryptone 10 g/L NaCl 10 g/L [pH was adjusted to 7.0 with 1N NaOH]
2.4.2. YPD Medium (Yeast extract Peptone Dextrose) (per 1000 mL)	Yeast extract 10 g/L Peptone 20 g/L Dextrose 20 g/L
2.4.3 SD Medium (Synthetic Defined) (per 1000 mL)	YNB(Yeast Nitrogen Base) 1.7 g/L (without amino acids and ammonium sulphate) $(NH_4)_2 SO_4$ 5 g/L Dextrose 20 g/L Amino acids (as required) 80 mg/L [pH was adjusted to 6.0–6.5]

2.5 BUFFERS AND STOCK SOLUTIONS

2.5.1 Ampicillin (100 mg/mL) stock solution

The required amount of ampicillin (sodium salt) was dissolved in the required volume of deionized water and was filter-sterilized using a 0.2µm membrane filter.

2.5.2 Chloramphenicol (50mM) stock solution

The required amount of chloramphenicol (solid form) was dissolved in 80ml of 100% ethanol and was filter-sterilized using a 0.2µm membrane filter. It was stored at -20°C in aliquots.

2.5.3 IPTG (1M) stock solution

Dissolve 2.36g of IPTG in 8mL of distilled water, and the final volume is made up to10 Ml. filter sterilize with a 0.2µm membrane filter. It was stored at -20°C in aliquots.

2.5.4 50% Glycerol (used for preparing –80°C stocks of *E. coli*)

50 mL glycerol is dissolved in 50 mL of deionized water and mixed properly. The solution was autoclaved and stored at room temperature.

2.5.5 25% Glycerol (used for preparing –80°C stocks of *S. cerevisiae*)

25 mL glycerol is dissolved in 75 mL of deionized water and mixed properly. The solution was autoclaved and stored at room temperature.

2.5.6 Alkaline Lysis Buffers (Plasmid DNA preparation from *E. coli*)

a) Solution-I (Resuspension Solution)	50 mM Glucose 25 mM Tris-HCl (pH 8.0) 10 mM EDTA (pH 8.0) Autoclaved and stored at 4ºC.
b) Solution-II (Lysis Solution) (freshly prepared)	0.2N NaOH (freshly diluted from a 10N stock)

	1% SDS (freshly diluted from a 10% stock) Stored at room temperature.
c) Solution-III (Neutralization Solution)	5M Potassium acetate 60 mL Glacial acetic acid 11.5 mL Deionized water 28.5 mL The resulting solution is 3M with respect to potassium and 5M with respect to acetate. It was stored at 4ºC.
d) TE Buffer (Tris-EDTA) **(pH 8.0)**	10 mM Tris-HCl (pH 8.0). 1 mM EDTA (pH 8.0).
e) TE-RNase **(stock 10 mg/mL)**	Working stock 20 µg/mL in TE Buffer, pH 8.0.
f) PCI (Phenol-chloroform-isoamyl alcohol) Solution (100mL)	a) Phenol 50 ml [Equilibrated with Tris-HCl (pH 7.6)] b) Chloroform 48 ml c) Isoamylalcohol 2 ml Stored at 4°C in a dark bottle.

2.5.7 Agarose Gel Electrophoresis Reagents

a) 1× TAE (Tris-acetate-EDTA) Buffer (per 1000 mL) **(prepared from 50× TAE stock)**	40 mM Tris-acetate. 1mM EDTA (pH 8.0). Autoclaved and stored at room temperature.
b) Orange-G dye (Gel loading dye, 6X)	0.25% orange-G 30% glycerol

c) 0.7-1% Agarose gel in 1× TAE	1% Agarose in 1X TAE
d) Ethidium Bromide (10 mg/mL) Stock	Final working concentration used at 0.5µg/mL.

2.5.8 Yeast Transformation Solutions (*S. cerevisiae*)

a) 10X Lithium acetate (per 1000 ml) (pH7.5)	100.2 g of lithium acetate dissolved in 900mL of deionized water. pH adjustedto7.5 with diluted glacial acetic acid and volume bought up to 1000mL. Autoclaved and stored at room temperature. It is autoclaved and stored at RT.
b) 10X Tris-EDTA(TE) (per 1000 ml) (pH7.5)	15.759 g of Tris-Cl and 2.92g of EDTA added to 800mL of deionized water. pH adjusted to 7.5 and volume bought up to1000mL. Autoclaved and stored at room temperature. It is autoclaved and stored at RT.
c) 50% PEG (Polyethylene glycol) (per 1000 ml)	500g of PEG3500 dissolved in 500mL of deionized water by vortexing. Volume made up to 1000mlby adding deionized water. It is autoclaved and stored at RT.

2.5.9 Solutions for preparation of chemically competent *E. coli* cells

a) SOB	Bactotryptone 20 g Bacto yeast extract 5 g NaCl 0.5 g Above mentioned components were dissolved in 950 mL of water. 10 mL of 250 mM KCl was added and pH was adjusted to 7 with 5N NaOH, volume was made up to 995 mL and autoclaved. Just before use, 5 mL of filter-sterilized 2 M $MgCl_2$ was added.

b) SOC	SOB + 20 mM Glucose
c) 10% glycerol	10 mL glycerol is dissolved in 90 mL of deionized water and mixed properly. The solution was autoclaved and stored at room temperature.

2.5.10 Solutions required for SDS PAGE

a) Stalking gel (5%)	0.5 M Tris-HCL at Ph 6.8 =1.25ml 10% SDS =50 µl Acrylamide (30% w/w) =670 µl TEMED =10 µl APS 10%= 50 µl d.water = 2.975 ml * components for 2 gel
b) Resolving gel (12%)	0.5 M Tris-HCL at Ph 8.8 =2.6 ml 10% SDS = 100 µl Acrylamide (30% w/w) = 4 ml TEMED = 20 µl APS 10% = 100 µl d.water = 3.2 ml * components for 2 gel
c) Running buffer (1X)	Tris base = 3 g Glycine = 14.4g SDS = 1.0 g Make up the volume to 1000ml. store at room temperature.
d) Staining solution	0.4g of coomassie blue R350 in 200 Ml of 50 % (v/v) methanol in water with 10 % (v/v) acetic acid. store at room temperature.
e) Destaining solution	50 % (v/v) methanol in water with 10 % (v/v) acetic acid. store at room temperature.

2.5.11 Solutions for protein purification

a) Lysis buffer	50mM tris and 300mM NaCl in water Tris = 9.0 g NaCl =17.5 g Volume is made up to 500ml and autoclaved Store at 4°C.
b) Imidazole	1M imidazole and pH is set to 8.0 with Con.HCL and stored in a dark bottle at room temperature.
c) PMSF	100mM Phenylmethylsulfonyl fluoride in Isopropanol 17.4 g PMSF in 1ml of isopropanol. Stored at -20°C.
d) Washing buffer	20mM Imidazole in lysis buffer (2.5.11a) Freshly prepared and kept in ice.
e) Elution buffer	250mM imidazole in lysis buffer (2.5.11a) Freshly prepared and kept in ice.

SECTION B: METHODS

2.6 Growth and maintenance of bacteria and yeast strains

The *E. coli* DH5αand BL21(DE3) pLysS strains were routinely grown in LB medium at 37°C. *E. coli* transformants were selected and maintained on LB medium supplemented with ampicillin and ampicillin along with chloramphenicol for DH5α and BL21(DE3) pLysS respectively.

The *S. cerevisiae* strains were regularly maintained on YPD medium and grown at 28-30°C. The yeast transformants were selected and maintained on SD medium with amino acid supplements as per requirements.

2.7 Recombinant DNA methodology (restriction digestion, ligation, *E. coli* transformation, PCR amplification, etc)

All the molecular techniques used in the study for manipulation of DNA, protein, bacteria and yeast were according to standard protocols or as per the manufacturer's protocol unless specifically mentioned. DNA sequencing was done using Agrigenome sequencing services.

2.8 Transformation of yeast

The transformation of *S. cerevisiae* strains was carried out by the lithium acetate method. *S. cerevisiae* cultures were grown in YPD at 30°C with shaking for 12-18hrs and then reinoculated in fresh YPD to an initial OD_{600} of 0.1, cells were allowed to grow at 30°C for 4-5 hrs with shaking. Cells were harvested at 6000 rpm for 5 min, then were washed with sterile water followed by subsequent wash with 0.1M lithium acetate solution (prepared in TE, pH 7.5) and were finally resuspended in the same solution. The cells were spun down, suspended in 0.1 M lithium acetate solution to a cell density of $1 \cdot 10^9$ cells/mL and divided into 100µL aliquots. Approximately 50 µg (5µL of 10 mg/mL stock solution) of heat-denatured, salmon sperm carrier DNA, 0.3µg- 0.7µg of plasmid/DNA fragment and 0.3mL of 50% PEG 3350 (prepared in 0.1M lithium acetate, pH 7.5) were added to each aliquot and the whole-cell suspension was subjected to heat shock at 42°C for 30 min. The cell suspensions were allowed to cool to room temperature. The cells were centrifuged at 9000 rpm for 5 min. The cell pellet was resuspended in sterile water and the appropriate volume of cell suspension was plated on selection plates.

2.9 Dilution spotting

For dilution spotting yeast cells were grown in the selective minimal medium. Primary cultures were grown for 12-16 hours at 30°C, with shaking at 200 pm. Primary cultures were used to inoculate a secondary culture in the selective minimal medium for 8 hours or till OD_{600} reaches between 0.6-0.8 at 30°C. Cells were centrifuged, washed with sterile water and resuspended into sterile water, at OD_{600}= 0.2.

Four serial dilutions were made in sterile water; $O.D_{600}$= 0.2, 0.02, 0.002, 0.0002. 10µl of each of these cell suspensions were spotted on the selective minimal medium plates. Plates were incubated at 30°C for 2-5 days and photographs were taken.

2.10 Plasmid isolation from *E. coli*

The cells were grown in LB medium having ampicillin antibiotic for 14-16 hrs. Centrifugation was performed at 6000 rpm for 5 minutes, and the supernatant was discarded. 100 µl solution 1 was added followed by gentle mixing and incubation in ice for 5 minutes. After that 200µl solution 2 was added and gently mixed by reverting the microcentrifuge tube and immediately 150µl solution 3 was added by mixing and kept in ice for 10 minutes. The cells were centrifuged at 11,000 rpm for 10 minutes, and the supernatant was collected. An equal volume of phenol-chloroform-isoamyl was added which was mixed by vortexing for 2 minutes, and centrifugation was performed at 13,000 rpm for 5 minutes. The aqueous phase was collected, and an equal volume of isopropanol was added followed by incubation at room temperature for 15 minutes. Centrifugation was performed at 13,000 rpm for 10 minutes, and the pellet was resuspended in 70% ethanol. Centrifugation was performed at 10,000 rpm for 5 minutes, and the supernatant was discarded followed by incubation at 37oC for 30 minutes. The pellet was resuspended in TE-RNAse, and the plasmids were stored at -20°C.

2.11 Transformation of *E. coli*

E. coli competent cells were prepared using the calcium chloride method following standard protocols. The transformation was carried out by adding plasmid or ligation mixture to the competent cells, incubated, followed by a thermal shock at 42 °C for 1 minute. Transformed cells were incubated in LB at 37 °C for 45 – 60 min, and plated on LB medium containing antibiotics.

2.12 Sequence analysis

Protein sequences were retrieved from NCBI and UniProt database and multiple sequence alignment was done using Clustal omega server and EMBOSS Needle for nucleotides. JalView software was used for visualization purpose. Protein structures were retrieved from the PDB database.

2.13 IPTG induction

In LB with suitable antibiotics, freshly transformed cells are grown for 16 hrs in 37°C 200rpm and secondary is made to 0.02 $O.D_{600}$ and grown for 2 to 3 hrs in 37°C 200rpm till the $O.D_{600}$ reached between 0.6-0.8. Add IPTG optimal and grow the cells in optimal temperature depending on the protein of choice. Pellet down the cells at 6000rpm for 5min and store pellet in -80°C. For optimizing the protein

induction different concentration of IPTG is used and grown in different temperature. The induction is visualized in SDS PAGE after lysing the cell.

2.14 His tagged protein purification using Ni-NTA beads.

IPTG induced cells stored in -80°Cis resuspended in lysis buffer with 1mg/ml Lysozyme and 10µl/ml PMSF 100mM. it is vortexed well and incubated in ice for 30 min. sonicate the sample in ice till cells are lysed properly (about 1:30 min in 10-sec pulse, 15-sec rest cycle with amplitude 2). The sonicated cells are then pelleted down at 8000-9000 rpm for 30 min at 4°C. the supernatant is mixed with 5mM imidazole and Ni-NTA beads which are prepared after washing twice with water and twice with lysis buffer at 1000rpm for 1 min. the sample is the kept for binding in a slow rotter for 1 hour. Calibrate a filter column and run the sample through (collect the flow-through FT). Wash the column with wash buffer(save the first wash W1), and then elute out the protein to small aliquots by elution buffer with 5 min incubation in ice. The presence of protein is visualized in an SDS-PAGE running flow through, wash1, and elutions.

2.15 SDS PAGE

For cell lysate, 1 $O.D_{600}$ cell pellet is mixed well with30 µl lower gel buffer and 15µl of SDS loading buffer and heat denature for 15 min in 95 °C. Spindown at 8000rpm for 5 min. A 10 µl of the sample is then loaded in SDS gel and ran at a potential of 80V for 3 hrs. the Gel is then retrieved stained by Coomassie brilliant blue stain and destained by destaining solution.

For protein samples, 10 µl sample is mixed with an equal volume of SDS loading buffer and boiled for 5 min in 95 °C. A 10 µl of the sample is then loaded in SDS gel and ran at a potential of 80V for 3 hrs. the Gel is then retrieved stained by Coomassie brilliant blue stain and destained by destaining solution.

2.16 Carotenoid screening

Carotenoids are isoprenoids and can be used as a measure of the flux in the isoprenoid pathway. The carotenoid screen involves the expression of carotenoid synthesis genes in *S.cerevisiae* to synthesize Lycopene, which gives the cells a red colour because of the lycopene accumulation in the cell. The GGPP produced by GGPPS is further converted into phytoene and then to lycopene by phytoene synthase and phytoene desaturase respectively. In this assay, dilution spotting is done and grown for 3-4 days and then pigment formation visually evaluated. (Fig. 2.1 A)

2.17 Fluorescence screening

Fluorescence screening is done by developing a protein with a GFP tag that can sense GGPP, and the presence of GGPP causes the protein to be degraded by cells own machinery. This means the more the amount of GGPP, the lesser is the fluorescence. Cells to be tested are grown primary 12 to 15 hrs, and a secondary culture of OD = 0.2 is made and grown for 6 to 8 hrs. 1 OD cells from this are taken and fluorescence is measured by flow cytometry. (for this study FACS is done in BD FACSAria III Cell sorter by BD Biosciences. (Fig. 2.1 B)

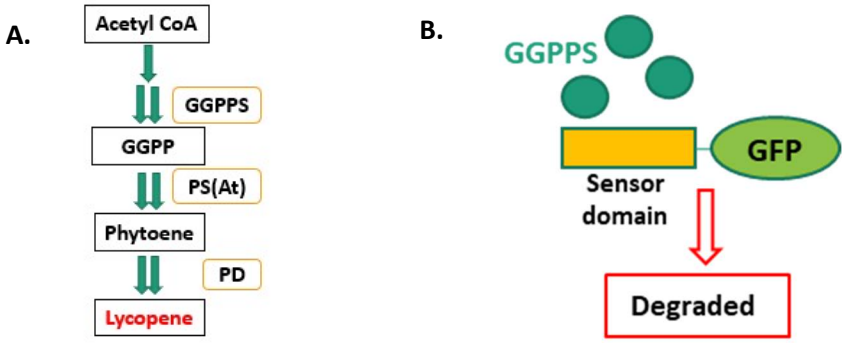


Figure 2.1: *A. Extending the MVP to produce red-coloured Lycopene. B. Schematic representation of GFP sensor protein degraded in presence of GGPPS*

Chapter 3: Results and Discussion

Section A: Results

3.1 *In-vitro* studies of the Fusion protein

A fusion protein of two consecutive enzymes of the pentose phosphate pathway was previously created in the lab. These were the fusions of Glucose 6-P dehydrogenase with 6-phosphoglucono-δ-lactone and involved 2 different fusions in which the order of the two proteins was reversed. The functionality of the fusion proteins under the TEF promoter was tested for complementation in Δzwf1 background. The Δzwf1 strain cannot grow without ZWF1 due to methionine auxotrophy. Here both fusions proteins and ZWF1 are able to complement the methionine auxotrophy (Fig. 3.1 A).

An *in-vivo* activity assay using a carotenoid screen was carried out to see how the carotenoid flux is different with fusion proteins. It is observed that there is no visual change in the colour (Fig. 3.1 B)

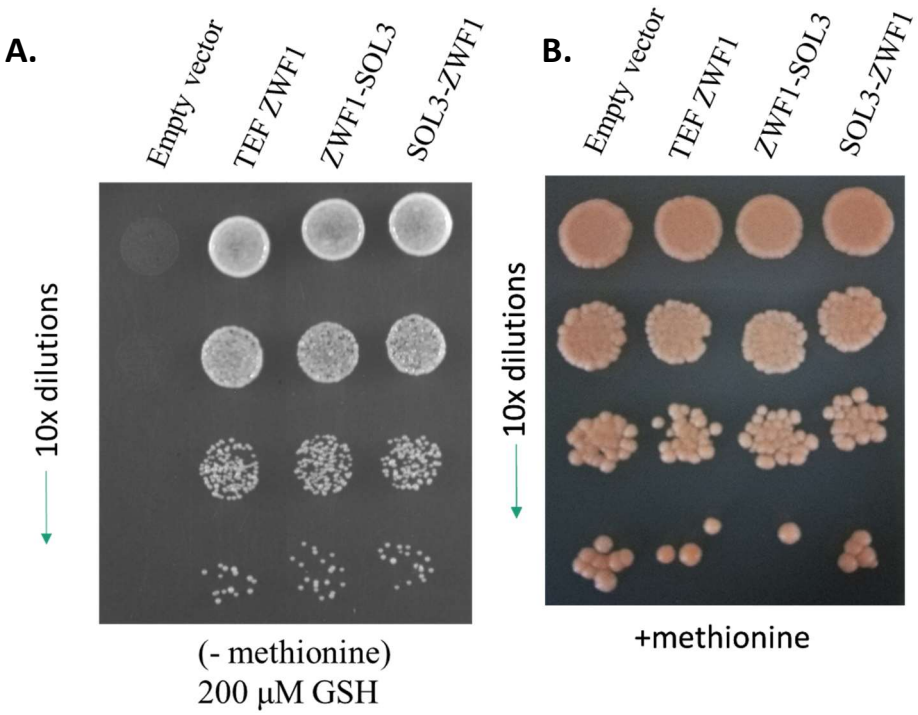


***Figure 3.1**: A. zwf1 screen shows complemention of Δzwf1. 200µM GSH was provided in the media as a sulfur source. B. Colour variations of the fusion protein in carotenoid screen (results adapted from Sri Harsha Adusumilli)*

3.2 Cloning and IPTG induction expression of fusion proteins and control ZWF1 protein

The two fusions were cloned into an *E. coli* expression vector as explained in the materials and methods. Small scale induction was done by varying the concentration of IPTG and changing the induction period by growing at different temperatures. Here IPTG induction is done on three different IPTG concentrations 0.0 (uninduced), 0.5, and 1.0 µM IPTG and three different temperatures 18, 30 and 37°C, where 18°C requires 16 hours, 30°C requires 6 hours and 37°C requires 3 hours induction time after IPTG addition. We were successful in inducing ZWF1 control protein and ZWF1-SOL3 fusion protein, and maximum induction was seen in 30°C and 0.5 µM IPTG (Fig. 3.2). These were the optimized conditions for large scale induction.

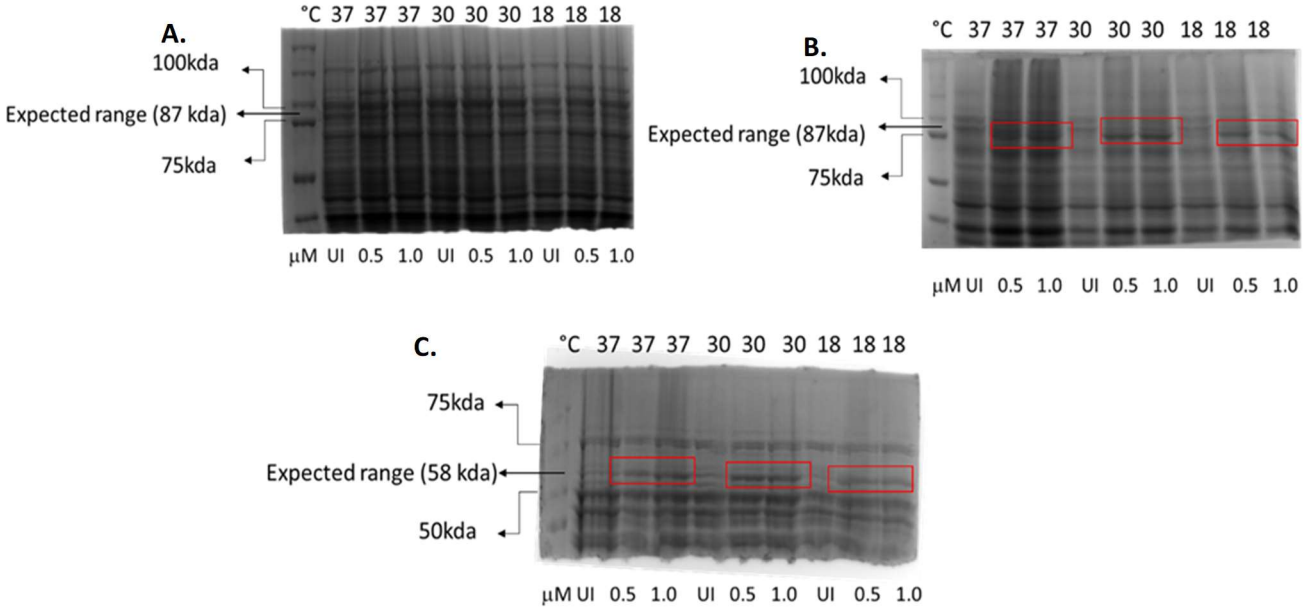


***Figure 3.2**: IPTG induction profile of **A**. sol3-zwf1 (failed to induce). **B.** ZWF1-SOL3 successfully induced at 87 kDa **C**. ZWF1 control successfully induced at 58 kDa. All proteins are expressed in three different IPTG concentrations 0.0 (uninduced), 0.5, and 1.0 µM IPTG and 18, 30 and 37 ℃ as shown and the red box indicate protein band induced in IPTG sample.*

3.3 Large scale induction and purification using Ni-NTA.

After optimising the induction conditions (0.5µM IPTG and induction at 30°C for 6 hours), large scale induction of 1 liter culture was perfomed and protein was purified using standard protocols. Ni-NTA beads were used to separate His-tagged protein. We were successful in purifying the ZWF1 protein and ZWF1-SOL3 protein with good concentration (Fig. 3.3).

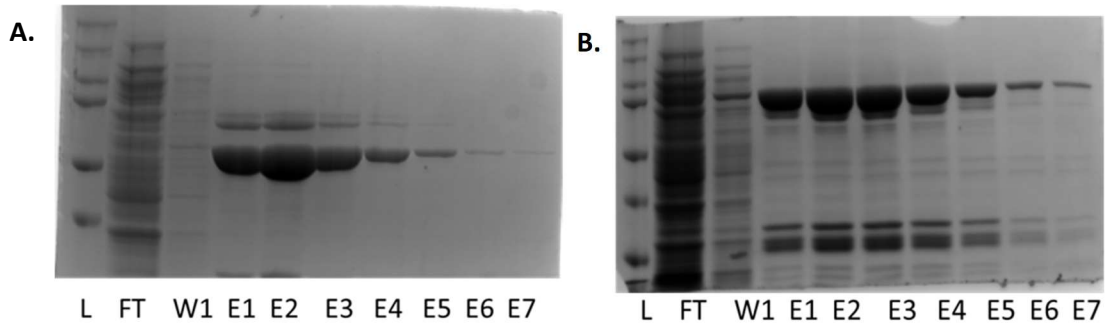
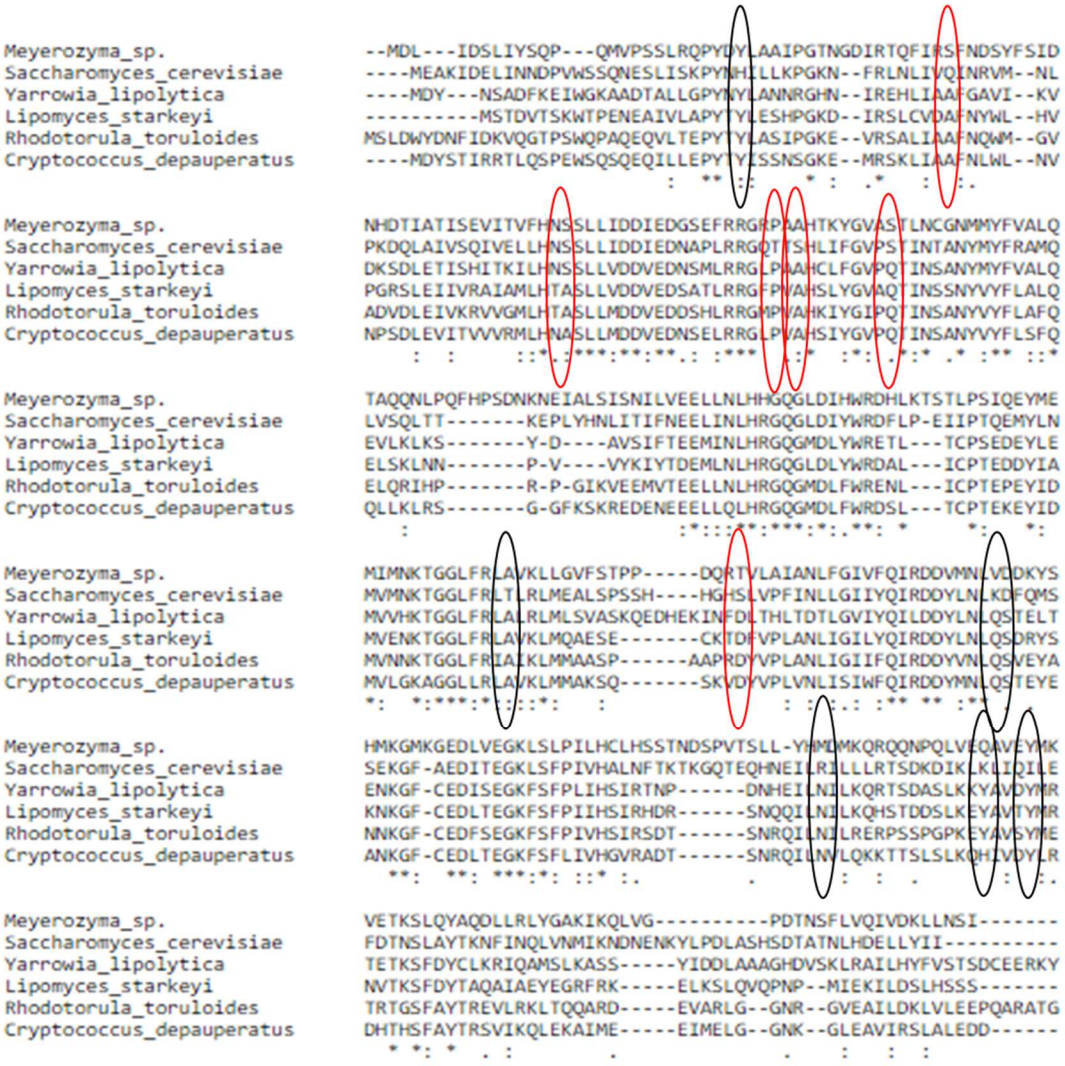


***Figure 3.3:** Protein purified visualised in SDS PAGE. **A**. ZWF1 at 58 kDa **B.** ZWF1-SOL3 at 87 kDa. Both gel contain ladder, flow through, first wash and seven elutions.*

3.4 Multiple sequence alignment of GGPPS

Multiple sequence alignment of *S. cerevisiae* GGPPS and Rt GGPPS along with other oleaginous yeasts reveals conserved amino acid residues in oleaginous yeast but absent in *S.cerevisiae* GGPPS (BTS1) see Fig. 3.4. To detect the conserved regions, the crystal structure data solved[11,12] for BTS1 and *Homo sapiens* GGPP was used. The amino acid residues conserved only in oleaginous yeasts were selected with their location in structurally and functionally important regions determined in previous studies.

A.



B.

M1: I 46 F	M2: S 70 A	M3: T 88 P + S 90 A	M4: S 98 Q	M5: S 193 D	M6:H 29 Y
M7: T 177 A	M8: R 259 N	M9: K 273 Y	M10: I 277 Y	M11: K 215 Q	M12: D 216 S

Figure 3.4: A. *Multiple sequence alignment of GGPPS of different yeasts; circles denote residues which are conserved in oleaginous yeast GGPPS and not in Sc GGPPS (red circles represent mutations selected for round one mutational studies)* ***B****. List of selected residues in Sc GGPPS (first a.a to be mutated, position on BTS1, mutated into a.a)*

3.5 Modelling of unknown Rt GGPPS structure and docking product

Since the crystal structure of Rt GGPPS is not solved as of now, we simulated a computational model for the Rt GGPPS to visualize the residues proximity to activity pocket and ligands binding sites. The structure modelled when superimposed with Sc GGPPS reveals the structural identity of both GGPPS (fig 3.5). We further docked product GGPP to the model and observed how much the mutations have the potential to interfere in enzyme catalysis (Fig. 3.5). This helped to further shortlist the potential mutatable residues and give confidence in continuing site-directed mutational studies.

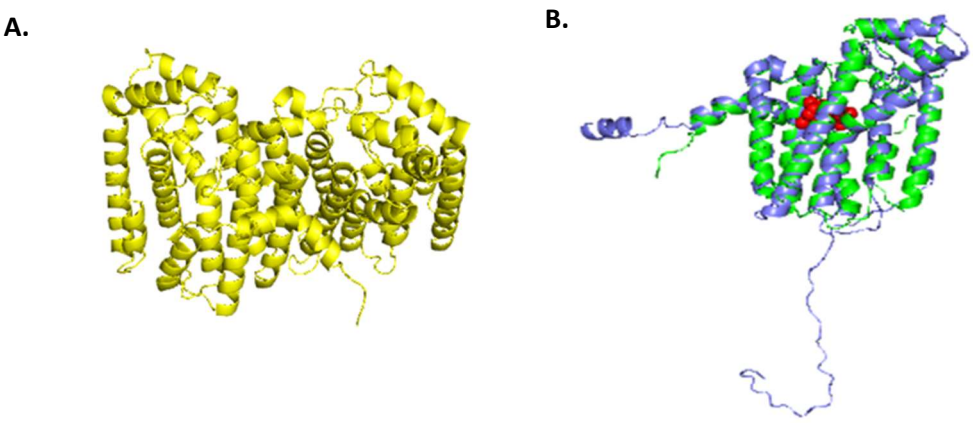


Figure 3.5: *A. Rt GGPPS modelled shown in dimeric form(yellow). B. Rt GGPPS (blue) superimposed with Sc GGPPS (green) in Pymol with ligand GGPP docked(red).*

3.6 Site-directed mutagenesis of BTS1 through Splice overlap extension (SOE) PCR

The primers with the mutation were used to create parts of the BTS1 and later the parts were mixed for the whole gene PCR giving the mutated BTS1. 5 mutations for round 1 were done, and combinations of these mutations were also made (Fig. 3.7) and in vivo studies were conducted.

Mutations on BTS1 selected for round 1 are:

M1 = I48F

M2 = S72A

M3 = T90P + S92A

M4 = S100Q

M5 = S195D

M1M2 = I48F + S72A

M4M5 = S100Q + S195D

MX5 = I48F + S72A + T90P + S92A + S100Q + S195D

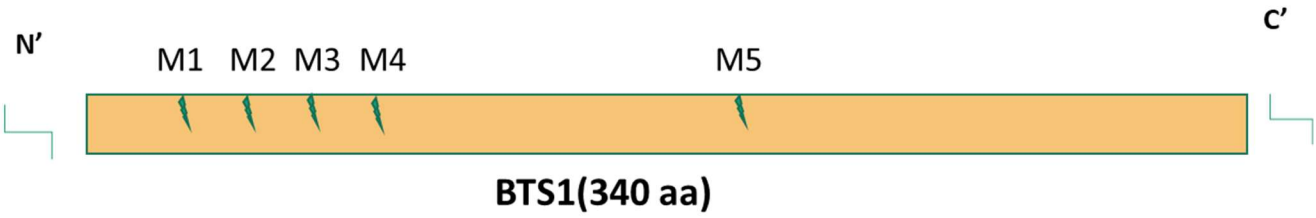


Figure 3.6: *Mutations selected for round 1 studies represented on a schematic of the BTS1 gene. Although they are depicted on the same figure, separate clones for individual and combination mutations were made.*

3.7 Carotenoid screen for BTS1 mutants

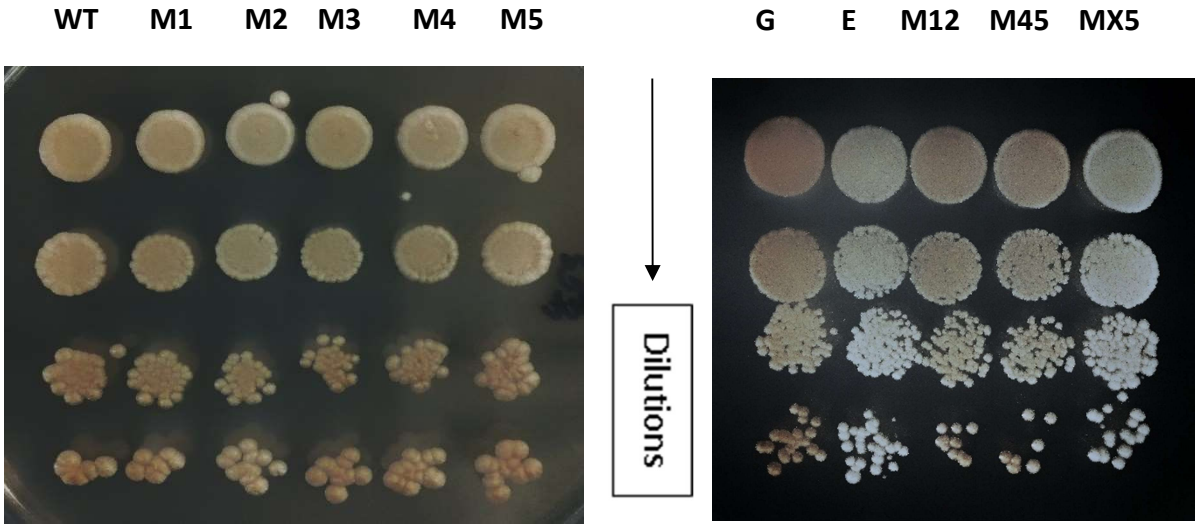


Figure 3.7: *ABC 733 strain transformed with pRS313TEF-BTS1 mutants and pRS416TEFPS(at)+PD show red colour. The transformants were grown in SD medium, and cells were harvested, washed, and resuspended to OD_{600} 0.2. Serial dilutions to OD_{600} 0.2, 0.02, 0.002, and 0.0002 were made, and 10 µl of each dilution was spotted onto SD Leu +Trp*

An *in-vivo* activity study is done to quantitate the activity of BTS1 mutations made. Along with double mutants (M12 with both M1 and M2 and M45 with both M4 and M5), all 5 mutations together is labelled MX5. The mutants were cloned in pRS313TEF vector and transformed into ABC 733 strain along with pRS416TEF-PS(AT)+PD plasmid for lycopene production. Selection was done on SD Met + Leu plate. Successful transformants were grown and dilution spotting was done to see their colour. A drastic colour difference was not observed for single and double mutations, but MX5 showed a loss of colour in the preliminary screen (Fig. 3.7), where G is the Rt GGPPS and E is the empty vector.

3.8 Fluorescent sensor screen for BTS1 mutants

Another in-vivo screening was the expression of GGPPS in a fluorescent sensor strain (Int-CEN.pk), which is a GGPP sensor stain developed in the lab (parallel work) that allows quantification of GGPP levels in the cell. Mutations were cloned in pRS313TEF and transformed in the fluorescent strain. Their median fluorescence was measured and plotted using the flow cytometry technique, which shows slight differences in GGPPS activity because of the respective mutations present in them (Fig 3.8)

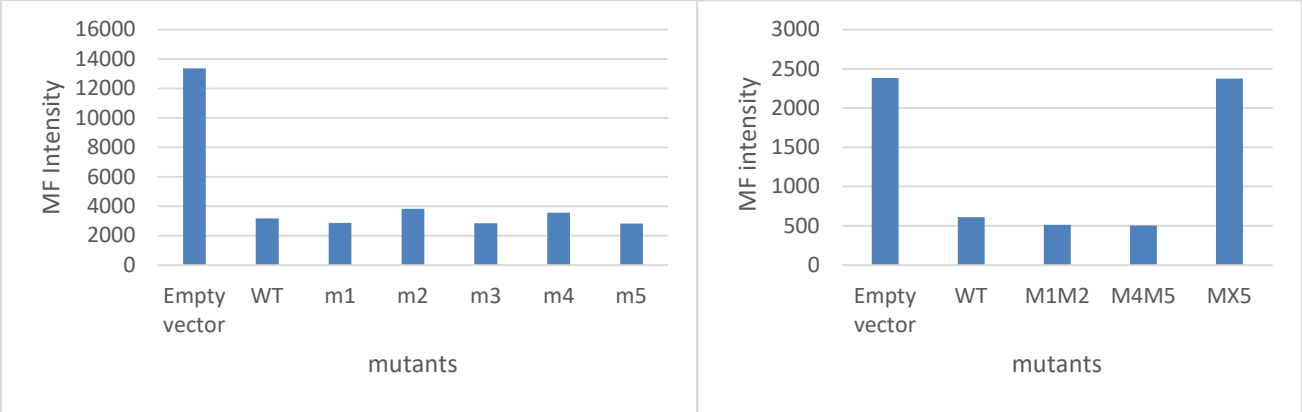


Figure 3.8: *Int-CEN.pk strain transformed with pRS313TEF- BTS1 mutants, selected in SD Trp+Leu, showing reduced fluorescence from empty vector due to GGPP production. Plotted median fluorescence intensity[MFI] of mutant BTS1. (**The experiment was done considering the median fluorescence of a single colony. Experiment with triplicate data is ongoing and the figure is to be replaced**.)*

Section B: Discussions

In this thesis, efforts were initiated to investigate two possible ways to target higher isoprenoid flux in *S. cerevisiae*. Two important enzymatic steps were targeted and two different approaches were taken for increasing the activity at each step.

In the first approach, we created fusion enzymes in PPP for a higher NADPH production, which is a key factor involved in the rate-limiting step of MVP, where HMG CoA is converted to mevalonate. The fusion enzymes were cloned and expressed, and in the *in-vivo* screen, it was observed that the fusions complement the ZWF1 deletion but the screens were not enough for quantitating the activity. So we proceeded for in vitro studies by purifying the proteins, where one fusion protein, which is N- terminal

ZWFI and SOL3 on C- terminal, was successfully purified using His-tag. The activity measurement is to be done in future work.

In the second approach, we looked at the GGPPS catalytic activity relative to superior variety seen in *Rhodosporidium toruloides*. We modelled and docked product GGPP on the Rt GGPPS computationally since the structure of Rt GGPPS was unknown, and with other species GGPPS crystal structure data solved previously[11,12]. We found potential amino acids playing a key role in the activity and mutated them on Sc GGPPS to enhance the activity. The mutants were screened in-vivo by two independent screens: (i) the carotenoid screen where more red colour shows higher GGPP production, thus efficient GGPPS, and (ii) the Fluorescent sensor screen, the lower the fluorescence observed, the higher the GGPP, the better the GGPPS. None of the screens showed much variation in the GGPPS efficiency from the wild type BTS1. But mutant MX5, where all the 5 mutations are incorporated seems to have lost its activity due to the combinatorial effect of mutations.

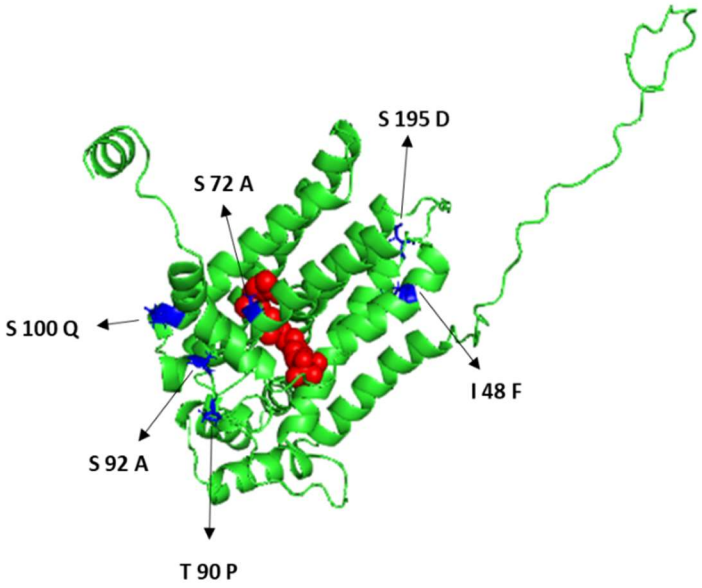


Figure 3.9: *Structure of BTS1-MX5 mutant (green) representing mutated residue (blue) labelled, and docked with the product GGPP (red).*

The mutated residues on MX5 are away from each other (Fig. 3.9). Since BTS1 with individual mutations shows activity, the reason behind the loss of activity of MX5 can be further investigated.

Finally, in addition to the *in-vivo* assays, in future work, the mutants that have to be purified and assayed *in-vitro* for their activity and the possible role of these residues in the functionality of the mutants are to be investigated further.

With the studies that have been initiated, it is hoped that in future, the lab would be able to make significant findings with this enzyme once the assays are carried out. The role of mutated amino acid residues in the mevalonate pathway flux will be interesting to determine.

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